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TITLE OF THE INVENTION (200 characters max.

SPECIFIC HIV GP120 CLEAVING ANTIBODIES INDUCED BY COVALENTLY REACTIVE ANALOG OF GP120 AND METHODS OF USE THEREOF

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☐ No

☒ Yes, the name of the U.S. Government agency contract number is: NIH- AI46029 and AI31268

Respectfully submitted
DANN, DORFMAN, HERRELL AND SKILLMAN
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PTO Reg. No. 43,047

☐ Additional inventors are being named on separately numbered sheets attached hereto

Specific HIV gp120 cleaving antibodies induced by covalently reactive analog of gp120 and methods of use thereof

Sudhir Paul
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Statement of the Invention

In accordance with the present invention, improved catalytic antibodies are provided which are obtainable via covelent reaction with a protein containing covalently reactive antigen analog. Such antibodies can be obtained from host cells or organisms expressing an antibody repertoire. They can also be obtained following conventional immunization protocols.

Catalytic antibodies so obtained may be used to advantage to treat a variety of disorders, including without limitation HIV infection, cancer and autoimmune disease.

Supported by NIH grants AI46029 and AI31268.

ABSTRACT

We report the results of efforts to strengthen and direct the natural nucleophilic activity of Abs for the purpose of specific cleavage of the HIV-1 coat protein gp120. Phosphonate diester groups previously reported to form a covalent bond with the active site nucleophile of serine proteases (Paul et al., *J. Biol. Chem.* 276, 28314, 2001) were placed on Lys side chains of gp120. Seven monoclonal Abs raised by immunization with this covalently reactive analog of gp120 (gp120-CRA) displayed irreversible binding to this compound (binding resistant to dissociation with the denaturant SDS). Catalytic cleavage of biotinylated gp120 by three MAbs was observed. No cleavage of albumin and the extracellular domain of the epidermal growth factor receptor was detected. Cleavage of model peptide substrates occurred on the C terminal side of basic amino acids, and Km for this reaction was ~200-fold greater than for gp120 cleavage, indicating Ab specialization for the gp120 substrate. A hapten phosphonate diester devoid of gp120 inhibited the catalytic activity with exceptional potency, confirming that the reaction proceeds via a serine protease mechanism. Irreversible binding of the hapten phosphonate diester by polyclonal IgG from mice immunized with gp120-CRA was increased compared to similar preparations from animals immunized with control gp120, indicating induction of Ab nucleophilicity. These findings suggest the feasibility of raising antigen-specific proteolytic antibodies on demand by covalent immunization.

Abbreviations. Ab, antibody; BSA, bovine serum albumin; Bt, biotin; CRA, covalently reactive antigen analog; L chain, light chain; MAb, monoclonal antibody; MCA, methylcoumarinamide; TSAs, transition state analogs; V domain, variable domain; VIP, vasoactive intestinal peptide

Promiscuous cleavage of small peptide substrates is a heritable function of Abs[1] encoded by germline gene V domains (reviewed in 1). Peptide bond cleaving Abs with specificity for individual polypeptides have been identified in patients with autoimmune (1) and alloimmune disease (2). Specific monoclonal Abs and Ab L chain subunits displaying proteolytic activities can be raised by routine immunization with polypeptides (3,4). Under ordinary circumstances however, adaptive maturation of the catalytic activity may not be a favored event. B cell clonal selection occurs by sequence diversification of genes encoding the Ab V domains, followed by selective binding of the antigen to cell surface Abs with the greatest affinity, which drives proliferation of the B cells (5). Catalysis entails chemical transformation of the antigen and release of products from the Ab, which may cause cessation of B cell proliferation when the catalytic rate exceeds the rate of transmembrane signaling necessary to stimulate cell proliferation.

Originally developed as irreversible inhibitors of conventional serine proteases, haptenic phosphonate esters are reported to bind the nucleophilic sites of natural proteolytic Abs covalently (6,7). The haptenic phosphonates could potentially serve as covalently reactive analogs (CRAs) for inducing the synthesis of Abs with improved nucleophilicity. To the extent that Ab nucleophilicity is rate limiting in proteolysis, its enhancement may permit more rapid peptide bond cleavage, i.e., if the subsequent steps in the catalytic reaction cycle (hydrolysis of the acyl-Ab complex and product release do not pose significant energetic hurdles; Fig 1). The innate character of Ab nucleophilic reactivity is the central element of this approach, and there is no requirement for *de novo* formation of chemically reactive sites over the course of V domain sequence diversification. Most previous attempts to program the structure of catalytic sites in Abs, in comparison, have relied on noncovalent stabilization of the oxyanionic transition state (i.e., by immunization with transition state analogs; 8,9). An Ab with esterase activity (10) and another with aldolase activity (11) utilize covalent catalytic mechanisms, but the relationship of these activities to innate Ab nucleophilicity is unclear.

An ideal antigen-specific proteolytic Ab may be conceived to combine traditional noncovalent binding interactions in the ground state of the Ab-antigen complex with nucleophilic attack on the peptide backbone. The ground state interactions are desirable to obtain specificity for individual polypeptide antigens. No impediments for catalysis are presented by the stable ground state complexes, provided the noncovalent interactions are carried over into the transition state complex and are properly coordinated with nucleophilic attack at the reaction center. In theory, synthesis of antigen-specific proteolytic Abs could be induced by an analog that presents a mimetic of the chemical reaction center in the context of classical antigenic epitopes available for noncovalent binding interactions. If the reaction proceeds by a lock-and-key stereochemical mechanism, the mimetic must be located precisely at the position of the intended scissile bond in the backbone of the polypeptide antigen. In the instance of large proteins, locating the mimetic within the protein backbone is outside the range of present-day synthetic technologies. A potential solution is to place the mimetic group at amino acid side chains using chemical linker techniques. An Ab nucleophile that recognizes the side chain mimetic could facilitate proteolysis if it enjoys sufficient conformational freedom to approach the polypeptide backbone of the substrate and form the acyl-Ab complex (Fig 1).

We describe here the characteristics of Abs induced by a covalently reactive analog (CRA) of the HIV-1 coat protein gp120 (gp120-CRA), consisting of phosphonate diester groups located in Lys side chains of the protein. Enhanced serine protease-like nucleophilic reactivity of the Abs was observed. One monoclonal Ab cleaved gp120 slowly and specifically, it displayed preference for cleavage on the C terminal side of Lys/Arg residues, and the catalytic reaction was susceptible to CRA inhibition. These findings are the first indications that Abs with proteolytic activity specific for individual proteins can be raised on demand.

MATERIALS AND METHODS

Hapten, gp120-CRAs and biotinylated proteins. Synthesis of hapten CRAs **I** and **II** (Fig 1) and their characterization by ESI-mass spectroscopy and elemental analyses have been described previously (12). For preparation of gp120-CRA **III**, the precursor diphenyl *N*-[O-(3-sulfosuccinimidyl)suberoyl]amino(4amidinophenyl)methanephosphonate **(IV)** was synthesized by mixing a solution of diphenyl amino(4-amidinophenyl)methanephosphonate (79 mg, 0.13 mmol) in DMF (2 ml) containing N,N-diisopropylethylamine (0.11 ml, 0.63 mmol) and bis(sulfosuccinimidyl)suberate disodium salt (150 mg, 0.26 mmol; Pierce) for 2 h. **IV** was obtained by reversed-phase HPLC (12) and lyophilized to give a colorless powder (yield 54%, 50 mg; m/z 715 (MH+) by electrospray ionization mass spectroscopy). **IV** (1.1 mg) was reacted with electrophoretically pure gp120 (0.5 mg; Immunodiagnostic Inc, MN strain, purified from baculovirus expression system) in 5 ml 10 mM HEPES, 25 mM NaCl, 0.1 mM CHAPS, pH 7.5 buffer (2 h, 25°C). Excess **IV** was removed by gel filtration (Micro Bio-Spin 6 disposable column, BioRad), and the concentration of free amines in the initial protein and CRA-derivitized protein was measured using fluorescamine (13). The density of labeling was varied as needed from 4.0 to 32.6 mol CRA/mol gp120 by varying the concentration of **IV**. Preparation of gp120 labeled at Lys residues with biotin (Bt-gp120) was by similar means using 6-biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Sigma). The reaction time and reactant concentrations were controlled to yield biotin/gp120 molar ratios 0.8-1.9. Unreacted biotinylation reagent was removed using a disposable gel filtration column in 50 mM Tris-HCl, 100 mM glycine, 0.1 mM CHAPS, pH 7.8. The biotin content was determined using 2-(4'-hydroxyazobenzene)benzoic acid (14). Total protein measurements were done using the BCA method (Pierce kit). Biotinylated **III** was prepared from Bt-gp120 as described for **III**. With increasing incorporation of the hapten groups, biotinylated **III** tended to form dimers and trimers evident in SDS-electrophoresis gels as bands at ~ 240 kD and 380 kD (nominal mass of monomer gp120, 120 kD). Biotinylated **III** at hapten density similar to the non-biotinylated **III** employed as immunogen

(23 mol/mol gp120) contained the monomer, dimer and trimer species at proportions of 50 %, 21 % and 29 %, respectively. Protein-CRAs were lyophilized and stored at –20°C until used. Bt-gp120 was stored at –70°C in 50mM Tris-HCl, pH 8.0, 0.1M glycine, 0.1mM CHAPS. Storage of **I** and **II** was at –70°C as 10 mM solutions in *N,N*-dimethylformamide. The extracellular domain of EGFR (exEGFR) obtained from Dr. Maureen O'Connor (15) was biotinylated as described for gp120 (0.9 mol biotin/mol exEGFR).

Antibodies. MAbs were prepared from female MRL/MpJ-*Fas*^{lpr} mice (Jackson Laboratory, Bar Harbor, Maine; 4–5 wk) immunized with gp120-CRA **III** (23 mol phosphonate diester/mol gp120). The mice were injected intraperitoneally on days 0, 14 and 28 days with gp120-CRA **III** (11 µg) in Ribi adjuvant (MPL+TDM emulsion; Sigma) followed by a fourth intravenous booster without adjuvant on day 55. Blood was obtained from the retroorbital plexus over the course of the immunization schedule. Three days following the final injection, hybridomas were prepared by fusion of splenocytes with myeloma cell line (NS-1; 3). Following identification of wells secreting the desired Abs by ELISA, monoclonal cell lines were prepared by two rounds of cloning by limiting dilution. Monoclonal IgG was prepared from tissue culture supernatants containing MAbs (200 ml) by affinity chromatography on immobilized Protein G (3). Control MAbs (anti-VIP clone c23.5 and anti-yellow fever virus antigen clone CRL 1689; ATCC) and serum IgG were purified similarly. The IgG preparations were electrophoretically homogeneous, determined by silver staining of overloaded IgG and immunoblotting with specific Abs to mouse IgG (3). Additional immunizations of female BALB/c mice (Jackson; 4–5 wk) with gp120 or gp120-CRA were carried out similarly. MAb heavy and light chain isotypes were determined by ELISA as described (3).

ELISA. Maxisorp 96-well microtitre plates (Nunc) were coated with gp120 or gp120-CRA (40-100ng/well) in 100 mM bicarbonate buffer, pH 8.6. Routine ELISAs were carried out as described (16). For assay of irreversible binding, the Abs were allowed to bind the plates and the wells were treated for 30 min with 2% SDS in 10 mM sodium phosphate, 137 mM NaCl, 2.7

mM KCl, 0.05% Tween-20, pH 7.4 (PBS-Tween) or PBS-Tween without SDS (control wells for measurement of total binding). The wells were then washed 3 times with PBS-Tween and bound IgG was determined as usual using a peroxidase conjugate of goat anti-mouse IgG (Fc specific; Sigma, Saint-Louis, MO). Observed values of binding were corrected for nonspecific binding in wells containing nonimmune IgG or nonimmune mouse serum (A490<0.03). Percent residual binding in SDS-treated wells was computed as: ($A_{490,\ SDS\ treated\ wells}$) x 100 / ($A_{490,\ PBS\text{-}Tween\ treated\ wells}$).

Electrophoresis of Ab-CRA complexes. Irreversible binding of biotinylated CRAs by purified IgG was determined by denaturing electrophoresis (6). Briefly, the reaction mixtures were incubated at 37°C in in 50mM Tris-HCl, 0.1M glycine pH 8.0. SDS was added to 2%, the mixtures boiled (5 min) and then subjected to SDS-PAGE (4–20%, Biorad, Hercules, CA; or 8–25% Phast gels, Amersham). Following electroblotting onto nitrocellulose membranes (0.22 µm, Biorad), the membranes were blocked with 5% skim milk in PBS-Tween and processed for detection of IgG or biotin using peroxidase conjugated goat anti-mouse IgG (Sigma) or peroxidase conjugated streptavidin, respectively. Imaging and quantification were using X ray film (Kodak) with Unscan-it software (Silk scientific, Orem, Utah) or a Fluoro-STM MultiImager (Biorad). Biotinylated BSA (11 mol biotin/mol BSA; Sigma) was employed to construct a standard curve (0.06–1.5 pmol biotin/lane).

Hydrolysis assays. Biotinylated proteins were incubated with IgG in 50mM Tris-HCl, 0.1M glycine, 0.1 mM CHAPS, pH 8 at 37°C, the reaction was terminated by addition of SDS to 2%, the samples were boiled (5 min) and then analyzed by reducing SDS-gel electrophoresis (4–20%, BioRad). Biotin containing protein bands in blots of the gel were identified and quantified as in the preceding section. In some blots, reaction products were identified by immunoblotting using peroxidase conjugated goat anti-gp120 Abs (Fitzgerald, Concord, MA; cat # 60-H14) (16). N terminal sequencing of protein bands from electrophoresis gels was done as described

previously (17). Hydrolysis of peptide-MCA substrates (Peptide Intn, Louisville, KY or Bachem Biosci., King of Prussia, PA) was determined in 96-well plates by fluorimetric detection of aminomethylcoumarin (Varian Cary Eclipse; λ_{ex} 360 nm, λ_{em} 470 nm) with authentic aminomethylcoumarin as standard (6). Cleavage of (Tyr^{10}-^{125}I)VIP by MAb c23.5 was measured as the radioactivity rendered soluble in trichloroacetic acid (17). Kinetic parameters for cleavage of increasing concentrations of peptide-MCA substrates were determined from the Michaelis-Menten equation: $v=(V_{max}[S])/(K_m+[S])$. Because of the expense of studying gp120 cleavage at large concentrations of the protein, K_d (~K_m) and k_{cat} for this reaction were obtained from the general quadratic equation (17): $[CS]^2 - [CS]([C_t] + [S_t] + K_d) + [C_t][S_t] = 0$, where $[C_t]$ and $[S_t]$ are the total concentrations of catalyst and substrate, and [CS] is the catalyst-substrate concentration. The method consists of calculation of [CS] at a series of assumed K_d values. The assumed K_d value yielding the best fit (by linear regression) between the observed reaction velocity and [CS] represents the experimentally determined K_d. k_{cat} is computed as the slope of the observed velocity versus [CS] plot.

RESULTS

gp120-CRA design and validation. Synthesis of hapten CRAs **I** and **II** (Fig 1) and their covalent reactivity with naturally occurring proteolytic Abs has been described previously (6,7). The electrophilic phosphonate mimics the peptide bond carbonyl group susceptible to nucleophilic attack, the positively charged amidino group adjacent to the phosphonate diester serves as a mimic of Lys/Arg P1 residues at which cleavage by germline encoded proteolytic Abs is observed (6), and the biotin group in **I** permits sensitive detection of Ab–phosphonate adducts. gp120-CRA **III** contains phosphonate diester groups in spatial proximity with antigenic epitopes presented by the protein. Multiple phosphonate diester groups were available per molecule of gp120, allowing presentation of the electrophilic hapten in conjunction with diverse antigenic epitopes.

Robust polyclonal Ab responses in MRL/lpr and BALB/c mice immunized with **III** were observed by routine ELISA. Abs raised to **III** were bound at somewhat greater levels by immobilized **III** than control gp120 devoid of phosphonate diester groups (Fig 2). Conversely, Abs raised to control gp120 recognized immobilized **III**, but the binding was 3-4 fold lower than by immobilized gp120 (e.g., at serum dilution of 1:1000, A490 0.44±0.03 for immobilized **III** and 1.40±0.03 for immobilized gp120). **III**-binding by nonimmune Abs was negligible, indicating that indiscriminate covalent binding at the hapten groups was not a problem. The observed differences in the antigenic reactivity of gp120 and **III** were held to be sufficiently small to proceed with further Ab studies. To facilitate high-throughput screening, the feasibility of measuring irreversible **III**-binding by Abs was studied by ELISA. Following binding of polyclonal Abs anti-**III** Abs to the immobilized antigens, ELISA plates were treated with the denaturant SDS to remove reversibly bound Abs. SDS treatment allowed essentially complete removal of anti-**III** Abs bound by control gp120 devoid of hapten phosphonate groups. In comparison, 13-40% of the overall anti-**III** Ab binding activity consistently remained bound to immobilized **III** following SDS treatment in 3 repeat experiments. SDS-electrophoresis and immunoblotting with Abs to mouse IgG confirmed formation of irreversible Ab-**III** complexes in boiled reaction mixtures (Fig 2 inset, lane 3, estimated mass from extrapolated standard curve of molecular mass standards, ~400 kD; large complexes can be formed by binding of multiple Abs to hapten groups in **III**).

Catalytic activity. MAbs were prepared from MRL/lpr mice immunized with gp120-CRA **III**. This mouse strain develops lupus-like autoimmune disease attributable to the dysfunctional Fas-receptor gene. Spontaneous development of proteolytic Abs (18) and increased synthesis of esterase Abs in response to immunization with phosphonate monoester haptens (19,20) have been reported in this mouse strain. Supernatants from 712 hybridoma wells (two splenocyte-myeloma cell fusions) were screened for SDS-resistant binding to **III**. IgG from seven wells was positive for this activity. Following cloning of the cells by limiting dilution, monoclonal IgG from the supernatants of the seven cell lines was purified and the binding assays were repeated (Fig

3; clones YZ 18, IgG2a,κ; YZ19, IgG2b,κ; YZ20, IgG2a,κ; YZ21, IgG2a,κ; YZ22, IgG2a,κ; YZ23, IgG2a,κ and YZ24, IgG1,κ). Of total binding observed without SDS treatment of the ELISA plates, residual binding following the detergent treatment was 43-83% in 4 repeat assays. All seven MAbs were also bound by gp120 devoid of hapten CRA groups determined by routine ELISA without SDS treatment, indicating that they are not directed to neoepitopes generated by chemical modification procedures used for III preparation. An irrelevant MAb (clone CRL 1689) displayed no detectable binding of III or gp120.

Of seven MAbs with irreversible III-binding activity, slow cleavage of Bt-gp120 by three MAbs was detected (YZ18, YZ20, YZ24), determined by appearance of biotin-containing fragments of the protein in SDS-electrophoresis gels. The electrophoretic pattern of Bt-gp120 cleaved by MAbs YZ18 and YZ24 were similar to that shown for MAb YZ20 in Fig 4. MAb YZ20 was studied further as it cleaved Bt-gp120 ~5 fold more rapidly than the other two MAbs. The consumption of gp120 was time dependent (Fig 4A). Major biotin-containing cleavage products with apparent mass 55 kD and 50 kD were observed, along with less intensely stained bands at 27 kD and 15 kD. A band at 35 kD was visible in overexposed gels, but this does not represent a product of MAb cleavage, as it was present at similar density in control incubations of Bt-gp120 in diluent. A control irrelevant MAb (clone CRL 1689) did not cleave Bt-gp120. Immunoblotting using polyclonal anti-gp120 Abs confirmed that non-biotinylated gp120 is also susceptible to cleavage by the MAb (55 kD cleavage product, Fig 4B). Both detection methods allow quantification of gp120 cleavage by measuring depletion of intact gp120. Neither method provides guidance about the complete product profile or product concentration, as Bt-gp120 contains minimal amounts of biotin (~1 mol/molgp120), and the polyclonal Abs used for immunoblotting do not react equivalently with the cleavage products.

MAb YZ20 did not cleave biotinylated BSA or the extracellular domain of the epidermal growth factor (exEGFR), indicating selectivity for gp120 (Fig 5A). Attempts to identify the bonds cleaved by MAb YZ20 were unsuccessful. N-terminal sequencing of the 55 kD and 50 kD bands yielded

identical sequences (TEKLWVTVYY), corresponding to the N terminal residues of gp120. Sequencing of the 15 kD band from the YZ20 reaction mixture did not yield detectable phenylthiohydantoin derivatives of amino acids, possibly due to a blocked N terminus. Identification of the 27 kD gp120 fragment is complicated because of its comigration with the Ab light chain in reducing gels. As identification of the precise bonds in gp120 cleaved by the MAb was not central to the present study, we turned to the use of model peptide substrates for determination of scissile bond preferences. A fluorimetric assay was employed to determine MAb-catalyzed cleavage of the amide bond linking aminomethylcoumarin to the C terminal amino acid in a panel of peptide-MCA substrates (Fig 5B). The peptide-MCA substrates are used at excess concentration (200 μM), permitting detection of even weakly cross-reactive catalytic Abs. Selective cleavage at Arg-MCA and Lys-MCA was observed, with no evident cleavage on the C terminal side of neutral or acidic residues. To confirm the absence of artifacts due to remote residues, we studied two tripeptide substrate identical in sequence except for the N terminal residue at the scissile bond, Gly-Gly-Arg-MCA and Gly-Gly-Leu-MCA. The former substrate was cleaved by Ab YZ20 and the latter substrate was not. The basic residue preference is consistent with the presence of positively charged amidino groups neighboring the phosphonate groups in the immunogen (**III**) and selective cleavage on the C terminal side of Arg/Lys residues by germline encoded proteolytic Abs observed previously (21,22).

Attainment of the desired catalytic properties, i.e., the ability to combine high affinity for individual antigens with rapid turnover, can be judged from the Km and kcat parameters (moles antigen cleaved/mol Ab/unit time). The Km of MAb YZ20 for Bt-gp120 was about 200-fold smaller than its preferred peptide-MCA substrate (E-A-R-MCA; Table I; single letter code for amino acids), consistent with development of specificity for gp120 by immunization with **III**. Twelve mol E-A-R-MCA were cleaved per mole MAb YZ20 over the course of the reaction (22 h), indicating that the MAb is capable of turnover, a defining feature of a catalyst. Turnover of Bt-gp120 was ~10-fold lower than of E-A-R-MCA. Previously, conventional non-Ab serine

proteases were reported to cleave short peptide more rapidly than large proteins (23), presumably because the former substrates are more readily accessible to the catalytic site.

Nucleophilic reactivity. gp120 hydrolysis by MAb YZ20 was inhibited by hapten CRA **II** (Fig 6), confirming the serine protease-like character of the MAb. **II** inhibition of MAb YZ20 cleavage of gp120 was 90-fold more potent than inhibition of MAb c23.5 cleavage of VIP (IC50, 0.4 μM and 36.0 μM, respectively). The latter MAb was obtained by immunization with VIP devoid of phosphonate diester groups (3). Superior reactivity of the hapten CRA with MAb YZ20 is consistent with the conclusion of strengthened Ab nucleophilicity in response to immunization with phosphonate groups present in the gp120-CRA immunogen.

To confirm induction of nucleophilicity, irreversible hapten CRA **I** binding by polyclonal IgG was measured. The hapten CRA does not contain antigenic epitopes belonging to gp120 and noncovalent binding interactions are not anticipated to contribute to its irreversible binding by Abs. IgG samples from all four mice immunized with **III** displayed superior **I** binding compared to IgG from mice immunized with control gp120 (mean values, 0.31 and 0.01 pmol **I**; $P<0.02$, Student's t test, unpaired observations) as well as pooled nonimmune IgG (Fig 6). BALB/c mice were studied in this immunization. It may be concluded that synthesis of nucleophilic Abs in response to immunization with **III** is not restricted to autoimmune hosts (MAbs to gp120-CRA **III** were prepared from MRL/lpr mice).

DISCUSSION

The goal of this study was to strengthen the intrinsic serine protease-like reactivity of Abs and direct the reactivity to cleavage of gp120. Improved irreversible binding of hapten CRA by Abs following immunization with gp120-CRA **III** was evident, and the hapten CRA was a potent inhibitor of gp120 cleavage by a MAb. These observations suggest adaptive improvement of Ab nucleophilicity induced by the phosphonate diester groups. Specificity of the Abs for gp120 was obtained by traditional noncovalent mechanisms, i.e., recognition of gp120 epitopes located in

the proximity of the phosphonate diester groups. No cleavage of unrelated proteins by the gp120 cleaving MAb was observed and the Km value of cleavage of a model peptide was 200-fold greater than of gp120 cleavage, indicating absence of indiscriminate proteolysis.

Proteolysis entails Ab attack on the backbone of gp120 whereas the phosphonate electrophiles are located in Lys side chains of the immunogen. As MAbs raised to gp120-CRA displayed proteolytic activity, the nucleophile developed to recognize the side chain electrophiles must enjoy sufficient conformational freedom to attack the polypeptide backbone. Movements of individual amino acids in Ab combining sites following binding to antigen have been reported (24,25). Epitope mapping and mutagenesis studies of certain proteolytic Abs have indicated that the catalytic residues do not participate in stabilization of the Ab-antigen ground state complex (26,27), suggesting that the mobility of the nucleophile may not be restricted by initial noncovalent Ab-antigen interactions. Naturally occurring MAbs to VIP (17) and gp41 (4) cleave multiple peptide bonds in these antigens, which may be explained by hypothesizing formation of alternate transition states in which the nucleophile is free to initiate nucleophilic attack on spatially neighboring peptide bonds (reviewed in 28). Understanding the extent of conformational freedom of Ab nucleophiles is important, as there is no viable alternative to locating the peptide bond mimetic in the side chains when large proteins must be used to induce the synthesis of catalytic Abs. In addition to direct structural analysis of nucleophile movements in available catalytic Abs, the length and flexibility of the linker utilized to attach the phosphonate groups at Lys side chains can be varied in future studies to assess the flexibility of the catalytic site. In the case of synthetic peptide immunogens, the phosphonate groups can be incorporated within the peptide backbone to better mimic the intended scissile bond (7). However, synthetic peptides often fail to assume conformations similar to their cognate determinants in full-length proteins, in which case anti-peptide Abs do not recognize the parent proteins.

The fully competent catalytic machinery found in modern non-Ab serine proteases has presumably evolved in response to selection pressures that optimized each of the rate limiting

steps in the catalytic cycle. In comparison, a CRA immunogen can at best select for Abs with the greatest covalent attack capability. No selection for hydrolysis of the acyl-Ab complex or the subsequent product release steps is anticipated, which may account for observations of limited Ab turnover. Two previous attempts to raise esterase Abs indicated the formation of irreversible substrate binding by Abs (29,30), suggesting the need to optimize events occurring after nucleophilic attack by the Abs. Further, structural refinements of the immunogen could be implemented to help guide the Ab-antigen complex towards the catalytic pathway, e.g., inclusion of a component that bind a water molecule and facilitate hydrolysis of the acyl-protein complexes. Notwithstanding this weakness, the results reported here represent a significant advance towards developing antigen-specific proteolytic Abs. Previously, several Abs with haptenic ester hydrolyzing activity have been raised based on the premise that catalytic sites capable of noncovalent stabilization of the oxyanionic transition states can be formed *de novo* over the course of adaptive sequence diversification of Ab V domains (8,9). This approach has not been successful for development of proteolytic Abs. Pollack and Schultz described the failure of a phosphonate monoester analog of Phe-Leu-Ala to induce proteolytic Ab synthesis (31). No attempt was made in this study to recruit the intrinsic properties of natural Abs for the purpose of protease synthesis, i.e., their nucleophilicity and selective recognition of basic residues adjacent to the cleavage site. Recently, phosphonate monoesters were discovered to form covalent bonds with nucleophiles in serine proteases, but their reactivity is weaker than the diester used in the present study, and no detectable reaction occurs unless an adjacent positive charge is present (6,12).

Evidence for increased potency due to the catalytic function has recently been published in regard to Ab antagonism of the biological effects of VIP, a 28-amino acid neuropeptide (32,33). Concerning gp120, a major hurdle has been to induce the synthesis of Abs that recognize the determinants involved in viral entry, i.e., the binding sites for host CD4 and chemokine receptors. Most Abs raised to monomer gp120 are directed to its variable region epitopes, and the Abs do

not neutralize diverse HIV-1 strains found in different geographical locations (34). Reversibly binding Abs must bind at or near the receptor binding sites of gp120 to sterically hinder HIV entry into host cells. Proteolytic Abs offer the potential advantage of gp120 inactivation even if cleavage occurs at a site that does not itself participate in binding to host cells. Discussion of the immunotherapeutic potential of MAbs to gp120-CRA III is beyond the scope of the present study, but initial HIV-1 neutralization studies suggest that certain MAbs raised to gp120-CRA III neutralizes HIV-1 primary isolate ZA009 (peripheral blood mononuclear cell cultures, infection measured by determining p24 antigen concentrations; Paul and coworkers, to be published elsewhere). A potential pitfall is that proteolytic Abs to monomer gp120-CRA may not recognize trimeric gp120 on the surface of HIV-1, as observed for reversibly binding Abs to the protein (35). The CRA immunogen techniques described in the present study are readily applicable to recently developed recombinant mimetics of trimeric gp120 (36), as well as whole HIV-1 particles.

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Table 1: Kinetic parameters for cleavage of Bt-gp120 and Boc-E-A-R-MCA by MAb YZ20. IgG (1μM) was incubated with Bt-gp120 (0.14 - 2.2 μM; 13h) or Boc-E-A-R-MCA 31 – 1000 μM, 6h). Cleavage of Bt-gp120 was determined by measuring depletion of the 120 kD intact protein band on SDS-gels run in duplicate, and of E-A-R-MCA, by fluorimetry in triplicate. Kinetic parameters for Bt-gp120 cleavage were computed using the general quadratic equation describing a one site binding interaction and for, E-A-R-MCA, by fitting the data to the Michaelis-Menten equation (see text).

Antigen	K_m, M	k_{cat}, min-1	k_{cat}/K_m, M^{-1} min^{-1}
Bt-gp120	2.0×10^{-6}	$3.4 \pm 0.1 \times 10^{-3}$	1.7×10^{3}
E-A-R-MCA	$4.0 \pm 1.2 \times 10^{-4}$	$3.3 \pm 0.4 \times 10^{-2}$	8.4×10^{1}

Legends

Fig 1: CRA structures (A) and their reaction with Abs (B). **III** is schematic representation of gp120 with R3 substituents at Lys residues. Left of **III** are streptavidin-peroxidase stained blots of SDS-electrophoresis gels showing biotinylated **III** containing 4 mol (lane 1) and 14 mol (lane 2) phosphonate diester groups/mol gp120. In **B**, ***Nu***, nucleophile; Ag'-Lys-OH, N-terminal antigen fragment; NH2-Ag", C-terminal antigen fragment; kcat=k3'+k3". A catalytic Ab forms the initial noncovalent complex by conventional epitope-paratope interactions. The active site nucleophile site attacks the carbonyl carbon of the scissile bond in Ag (substrate) to form the tetrahedral transition-state complex. The C-terminal antigen fragment is released and the acyl-Ab complex is formed. Hydrolysis of the acyl-Ab complex results in release of the N-terminal antigen fragment and regeneration of the catalytic Ab. The reaction with phosphonate-containing Ag recapitulates the interactions in the ground and transition state Ab-Ag complexes (noncovalent binding at peptide epitopes and nucleophilic attack by the Ab) but unlike the acyl-Ab intermediate, the phosphonyl-Ab adduct is a stable product. A potential weakness is that immunogen **III** does not contain structural feature favoring synthesis of Abs capable of rapid hydrolysis of the acyl-Ab intermediate and product release (bottom reaction scheme).

Fig 2: Irreversible III binding by polyclonal Abs. **A,** Immobilized gp120. **B,** Immobilized **III.** Shown are ELISA values for binding of polyclonal Abs in serum of mice hyperimmunized with **III** (pooled sera, N= 4 mice). Binding of nonimmune mouse serum was negligible (A490 of 1:100 nonimmune serum in A, 0.001; in B, -0.002). Residual and total binding represent A490 values in wells treated with and without SDS, respectively. ***Inset,*** Anti-IgG stained blot of SDS-electrophoresis gels showing **III** (0.3 μM) treated for 48 h with nonimmune IgG (lane 2, 0.1 μM) and anti-**III** IgG (lane 3, 0.1 μM). Large Ab-containing adducts are evident at ~ 400 kD in lane 3. Lane 1 is a shorter exposure of lane 2 showing a well-defined 150 kDa band at the position of the smear evident in overexposed lanes 2 and 3.

Fig 3. Irreversible III binding by monoclonal Abs. ELISA showing SDS-resistant **III** binding by tissue culture supernatants containing MAbs (YZ series) **(A)** and monoclonal IgG purified from clone YZ18 **(B)** raised by immunization with gp120-CRA **III**. MAb CRL1689 is an irrelevant monoclonal IgG with same isotype as MAbs YZ21 and YZ23. Immobilized antigens, gp120 and **III**. SDS-resistant **III** binding indicated by bars and curve labeled **III** + SDS.

Fig 4: Cleavage of Bt-gp120 by MAb YZ20. **A,** Streptavidin-peroxidase stained blot of SDS-electrophoresis gels showing time dependent Bt-gp120 cleavage by MAb YZ20 and lack of cleavage by MAb YZ19 (22 h incubation). IgG, 1 μM; Bt-gp120, 0.2 μM. OE, Overexposed lanes showing Bt-gp120 incubated for 22 h in diluent and with YZ20 IgG (1 μM). Product bands at 27 kDa and 15 kDa are visible in addition to the major 50-55 kDa bands. **B,** Anti-gp120-peroxidase stained blot of SDS-electrophoresis gel showing gp120 (1 μM) incubated with diluent or YZ20 IgG (1μM, 24 h).

Fig 5. Lack of cleavage of Bt-BSA and Bt-exEGFR by MAb YZ20 (A) and preferential cleavage at basic residues (B, C). **A,** Strepavidin-peroxidase stained blots of biotinylated proteins (0.2 μM) incubated with MAb YZ20 (1 μM, 24h). **B,** Fluorimetric determination of MAb YZ20 (0.5 μM) catalyzed cleavage of peptide-MCA substrates (200 μM, 22h). **C,** Cleavage of GGR-MCA and GGL-MCA by MAb YZ20 (0.5 μM). Concentration of both substrates was held at 12.5 μM because of limited solubility of Gly-Gly-Leu-MCA. Blocking groups at the N termini of the substrates were: succinyl - AE-MCA, AAA-MCA, AAPF-MCA, IIW-MCA; t-butyloxycarbonyl – EKK-MCA, VLK-MCA, IEGR-MCA, EAR-MCA; benzyloxycarbonyl – GGR-MCA, GGL-MCA.

Fig 6: Enhanced hapten CRA I covalent binding by polyclonal IgG from mice immunized with III (A) and potent inhibition of MAb YZ20 cleavage of Bt-gp120 by hapten CRA II (B). **A,** Binding of hapten CRA **I** (10 μM) determined by incubation with IgG (0.4 μM) from BALB/c mice immunized with **III** (Ms9-12) or control gp120 (Ms1-4) for 60 min, SDS-electrophoresis and quantification of the biotin-containing band at 150 kDa, NI, nonimmune IgG (pooled from 20

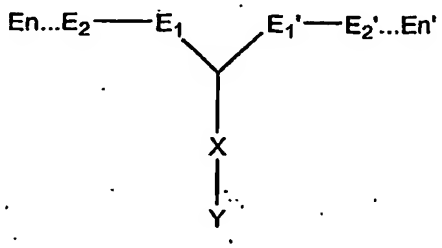
mice). **Inset,** Representative SDS-electrophoresis lanes showing hapten CRA **I** binding by IgG from a mouse immunized with **III** (Ms11), a mouse immunized with gp120 (Ms3) and nonimmune IgG (NI). **B,** Bt-gp120 (0.1μM) cleavage by MAb YZ20 (1μM, 4 h) and (Tyr^{10}-^{125}I)VIP (~100 pM, 45 K c.p.m.) cleavage by MAb c23.5 (20 nM, 18 h) was measured in the presence of increasing **II** concentrations. In the absence of **II**, 15% and 40% of available Bt-gp120 and VIP, respectively, were cleaved.

Fig 7A: Depicted is the noncovalent recognition between a pCRA and an antibody which occurs in conjunction with covalent recognition (shown by arrow) of the electrophile Y by the antibody nucleophile Nu. pCRAs of the present invention, as shown, contain the essential elements X, Y, E1 through En (E1...En), and E1' through En' (E1'...En') wherein n can be from 1 to approximately 30. Y is an electrophilic reaction center designed to react covalently with nucleophilic side chains of certain amino acids. Examples of Y include, but are not limited to: phosphonate, carbonyl, and boronate moieties. X is a component of the protein that contains a conjugatable group to which Y can be directly attached or attached through an adaptor. Typically, X is the side chain of any amino acid that includes a functional group (e.g., -NH2, -COOH, -SH, -OH). E1...En and E1'...En' are components of the antigenic epitope that make contact with the antibody noncovalently. E1...En and E1'...En' can be linear or discontinuous sets of amino acids. The dotted lines connecting E1...En and E1'...En' can be short or extended lengths of the polypeptide backbone that do not serve as components of the antigenic epitoipe. Generally, E1...En and E1'...En' are spatially neighboring components of an epitope within approximately 1 and 25 Angstrom of each other. Together E1...En and E1'...En' can be as small as 4 amino acids or as large as 30 amino acids.

Fig. 7B: Schematic drawings of two examples of X-Y combinations.

What is claimed is:

1. A catalytic antibody or component thereof obtainable through covalent interaction with a protein covalently reactive antigen analog (pCRA), comprising the following structural formula:



wherein Y is an electrophilic reaction center, X is a conjugatable group of the protein, and E1 through En and E1' through En' are components of the antigenic epitope.

2. The antibody of claim 1 which cleaves gp120.

3. The antibody of claim 2 which is an ScFv.

4. The antibody of claim 2 which is a light chain.

5. A humanized antibody comprising the Ag binding site of the ScFv of claim 3.

6. A humanized antibody comprising the Ag binding site of the light chain of claim 4.

7. The antibody of claim 1, obtainable via immunization with a pCRA.

8. The antibody of claim 7, wherein said immunization is performed on a host animal expressing a human antibody repertoire.

9. The antibody of claim 1, obtained by covalently reacting a pCRA with an antibody repertoire displayed on a host organism.

10. The antibody of claim 9, wherein said host organism is selected from the group consisting of yeast, bacteria and viruses.

11. The antibody of claim 10, wherein said host organism is a phage.

12. A method for immunizing a host organism, said method comprising administration of a pCRA to said organism to generate a protective catalytic antibody response.

13. The method of claim 12, wherein antibody catalyzes the cleavage of an HIV coat protein.

14. The method of claim 13, wherein said HIV coat protein is gp120.

Fig 1

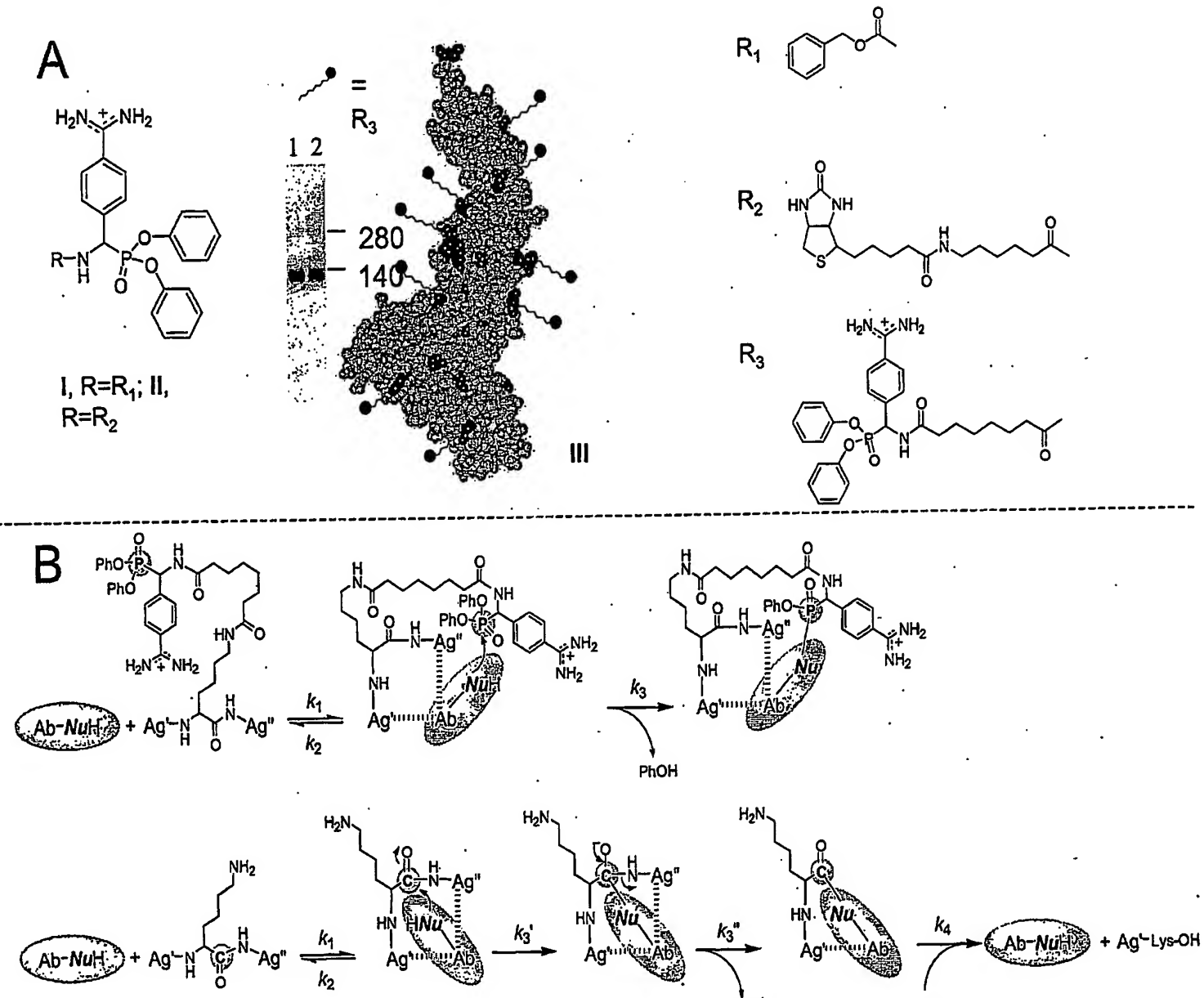

A
I, R=R1; II, R=R2
1 2
280
140
III
=
R3
R1
R2
R3
B
Ab-NuH
Ag'-Lys-OH
PhOH
H2O
H2N-Ag"

Fig 2

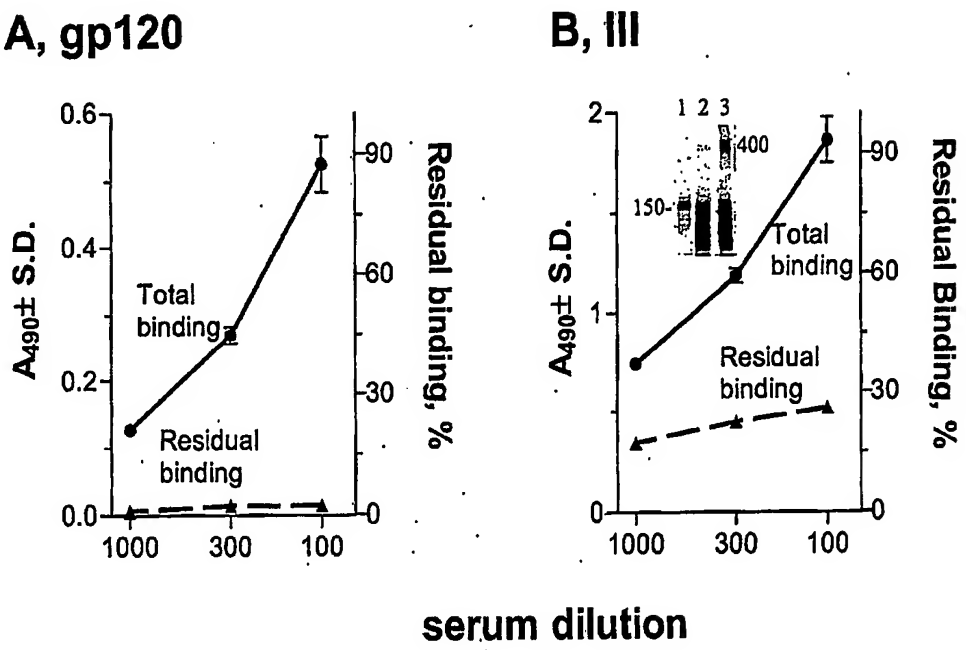

A, gp120
B, III
A_{490}± S.D.
Residual binding, %
Total binding
Residual binding
0.6
0.4
0.2
0.0
90
60
30
0
1000
300
100
2
1
1 2 3
400
150-
serum dilution

Fig 3

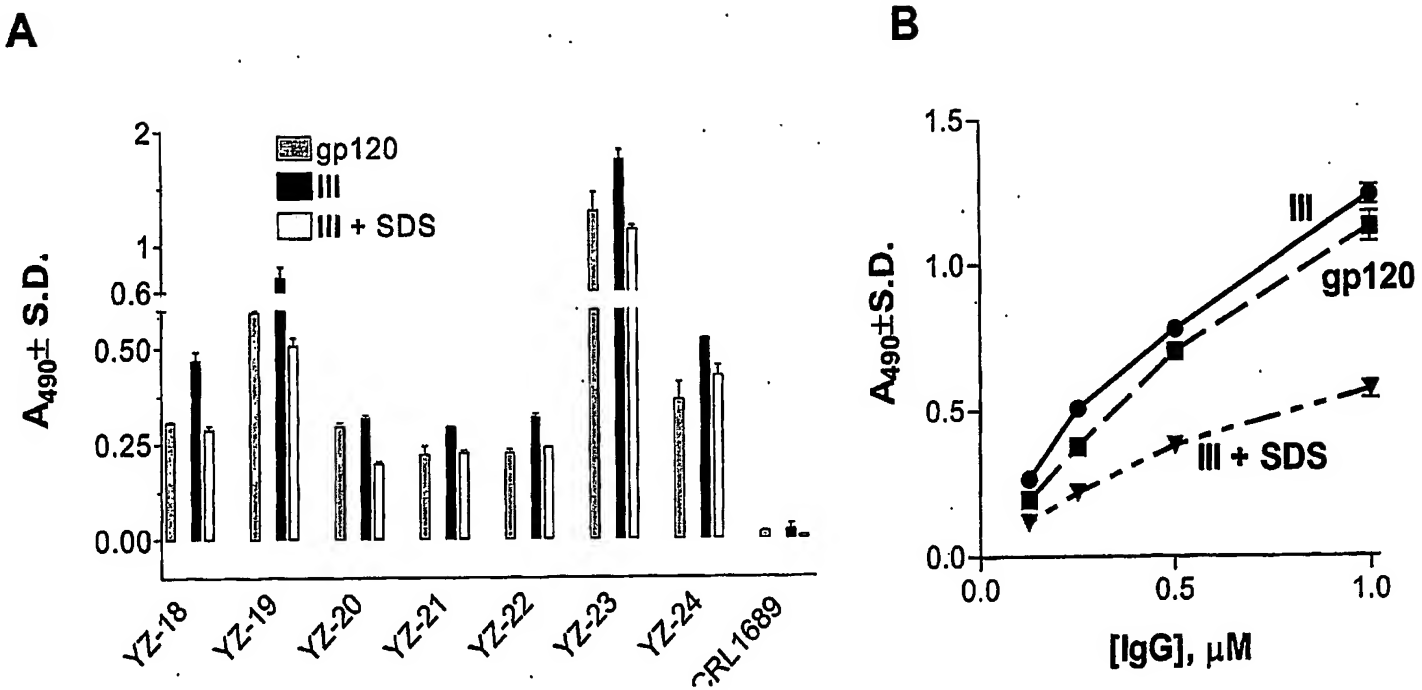

A
B
gp120
III
III + SDS
A_{490}± S.D.
2
1
0.6
0.50
0.25
0.00
YZ-18
YZ-19
YZ-20
YZ-21
YZ-22
YZ-23
YZ-24
CRL1689
1.5
1.0
0.5
0.0
0.0
0.5
1.0
[IgG], μM

Fig 4

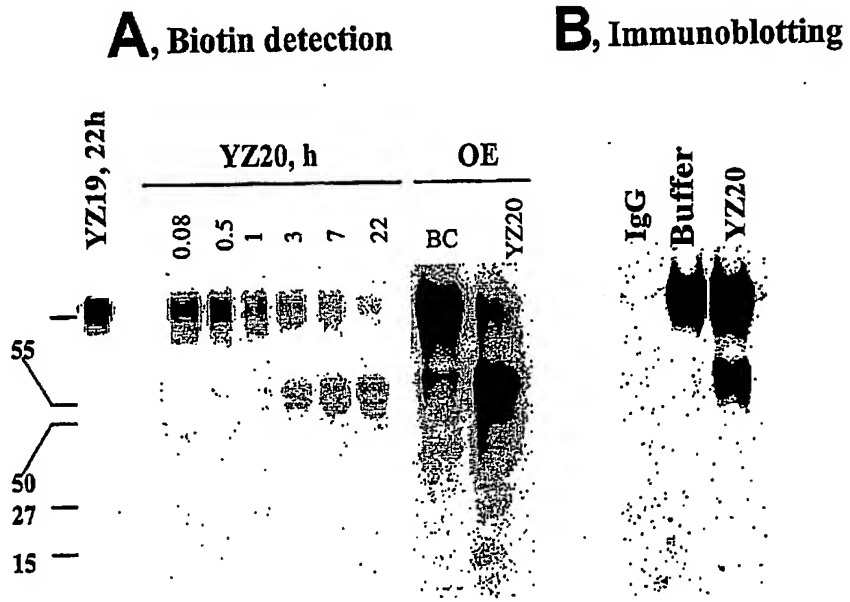

A, Biotin detection
B, Immunoblotting
YZ19, 22h
YZ20, h
OE
0.08
0.5
1
3
7
22
BC
YZ20
IgG
Buffer
YZ20
55
50
27
15

Fig 5

A
Bt-exEGFR +YZ20
Bt-exEGFR
Bt-BSA +YZ20
Bt-BSA
Bt-gp120 +YZ20
Bt-gp120
55
50
B
μM AMC / 22h / μM IgG
15
10
5
0
AE-MCA
AAA-MCA
IIW-MCA
AAPF-MCA
EKK-MCA
VLK-MCA
EAR-MCA
IEGR-MCA
PFR-MCA
C
μM AMC / 19h / μM IgG
0.4
0.3
0.2
0.1
0.0
GGR-MCA
GGL-MCA

Fig 6

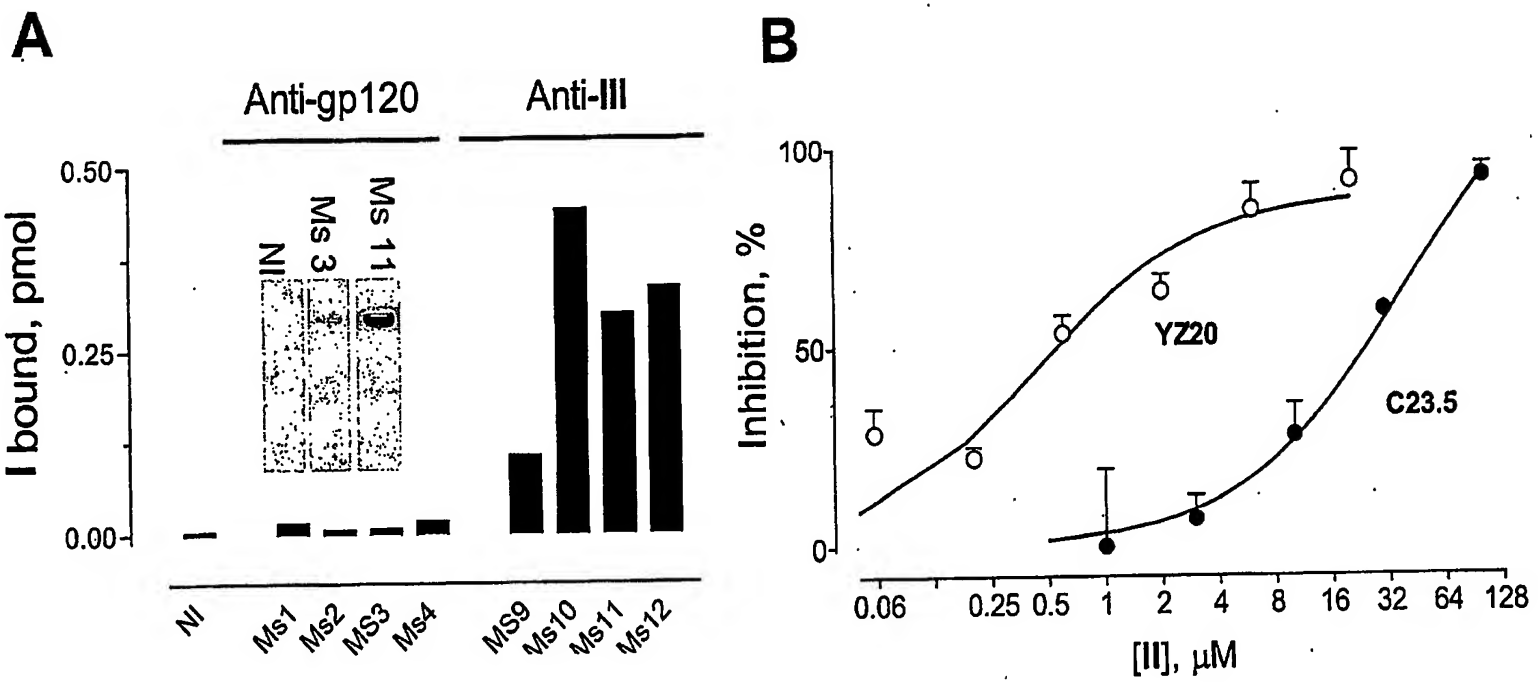

A
Anti-gp120
Anti-III
I bound, pmol
0.50
0.25
0.00
NI
Ms 3
Ms 11
NI
Ms1
Ms2
MS3
Ms4
MS9
Ms10
Ms11
Ms12
B
Inhibition, %
100
50
0
YZ20
C23.5
0.06
0.25
0.5
1
2
4
8
16
32
64
128
[II], µM

Fig. 7A

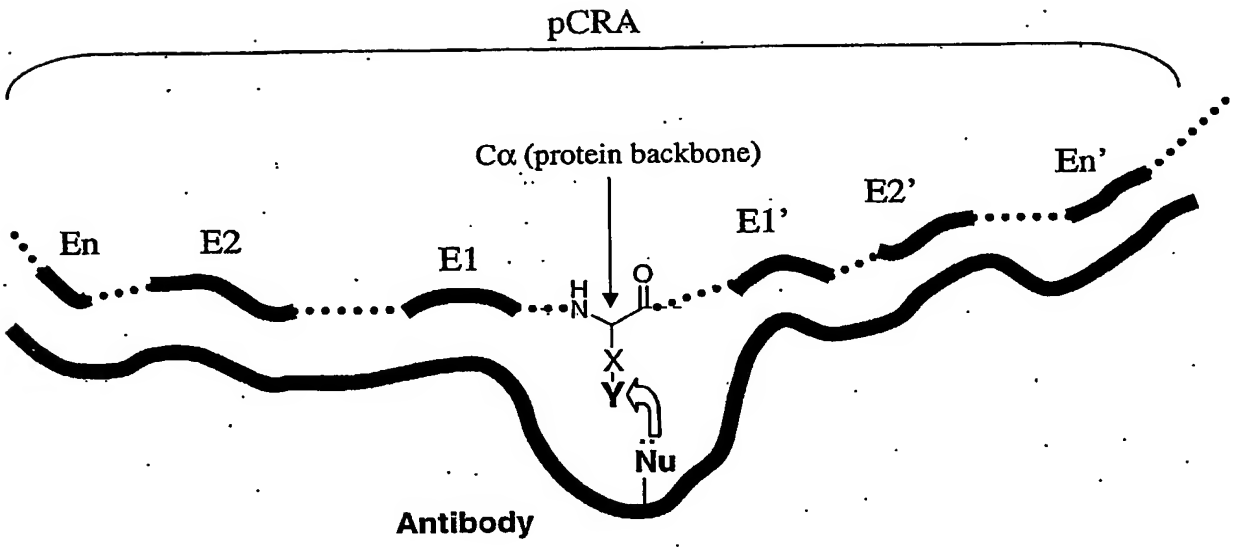
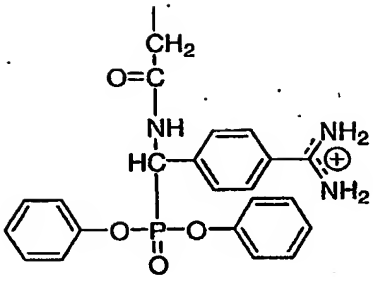


Fig. 7B

X-Y pair, Example 1:

X = Asp sidechain

Y = diphenyl amino(4-amidinophenyl)-methanephosphonate



X-Y pair, Example 2:

X = Lys sidechain

Y = diphenyl N-(suberoyl)amino(4-amidinophenyl)-methanephosphonate